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REMARKS

The Examiner's attention to the present application is noted with appreciation.

Applicant affirms the election to prosecute the invention of Group I, claims 1-22. The claims withdrawn from further consideration will be canceled in due course.

Applicant notes the informalities objected to by Examiner, and has tendered amendments relating thereto. Applicant noted additional typographical errors, and these are also the subject of amendments. No new matter is introduced thereby.

With respect to the obviousness type double patenting, Applicant will submit a terminal disclaimer upon allowance.

Claims Rejection - 35 U.S.C. § 112 - First Paragraph

Claims 1-22 are rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. For the reasons set forth below, Applicant respectfully traverses the rejection.

Applicant initially notes that claims 1-22 are directed to "method of treatment of neurologic damage". Much of the argument of the Examiner is directed to an alleged failure to prove that the method results in a cure. Here, the efficacy of the drug in animal studies was shown to (1) result in at least transient improvement in objective symptoms and (2) substantially delay onset of objective symptoms of genetically-induced disease. Both points are discussed in greater detail below, and in the Declaration submitted herewith. Such end points are directly related to a "method of treatment of neurologic damage". There is no requirement that a "method of treatment" result in an absolute cure.

The Examiner argues that "there is no data regarding the administration of thrombopoietin resulting in the expression of PDGF..." As to this point, Applicant maintains that it is known in the art that thrombopoietin increases platelet production, which necessarily results in increased expression of PDGF which are produced by platelets. Applicant so states at page 2, lines 23 - 25. That thrombopoietin is

known to increase platelet production, and doses of administration to effect increased platelet production, are generally taught in U.S. Patents Nos. 5,795,569; 5,879,673; and 5,989,537. Each of the foregoing U.S. patents is disclosed in the above-identified patent application, and is incorporated by reference therein. See also the Declaration of the inventor, George R. Schwartz, ¶ 3, submitted herewith.

With respect to the Examiner's comment on a perceived lack of data that administration of thrombopoietin results in expression of PDGF, thereby causing regeneration and repair of damaged neurons, Applicant notes that he is not required to prove how or why an invention works. *In re Spada*, 911 F.2d 705, 709, 15 USPQ2d 1655, 1657 (Fed. Cir. 1990). There is no enablement requirement that Applicant validate and offer data on each step of a proposed hypothesis or mechanism of action.

The Examiner notes that the examples do "indicate a delay on the symptoms of illness." However, the Examiner claims that the effect is "not clearly illustrated since there is no placebo group to compare with." As is set forth in the Declaration of the inventor, George R. Schwartz, ¶ 5, in this specific animal model the absence of a control group is of no import. First, as discussed in the declaration, in part each animal serves as a control for related experiments. Second, in the animal model the disease progression is known to be "progressive and without spontaneous improvement unless an effective therapy is initiated." Thus the nature of the animal model is such that no placebo is required. Further, it is established that there is no patentability requirement of a control or placebo. See, e.g., *Campbell v. Wettstein*, 476 F.2d 642, 177 USPQ 376 (C.C.P.A. 1973) (no control in animal experiments to inhibit pregnancy).

With respect to the asserted "undue experimentation", Applicant notes that the case cited by Examiner, *In re Wands*, 858 F.2d 731, 8 USPQ2d 1400 (Fed. Cir. 1988) involved significantly complicated experimentation to fuse and screen IgM antibodies for a particular claimed purpose. However, the court in *Wands* specifically held that the extensive experimentation required was nonetheless not "undue experimentation." Here, as in *Wands*, there is a high level of skill in this art at the time the application was filed, and as in *Wands*, all of the methods need to practice the invention are well-known. See also *Ex parte D*, 27 USPQ2d 1067, 1069 (B.P.A.I. 1993). Thus as *Wands* itself stated, "[t]he test is not merely

quantitative, since a considerable amount of experimentation is permissible, if it is merely routine..." Experimentation in terms of dosage, dose schedule, evaluation of endpoints and the like, which presumably are the factors to which Examiner refers, are well known in the arts of pharmacology, medicinal chemistry and clinical medicine.

Claims Rejection - 35 U.S.C. § 112 - Second Paragraph

Claims 1-22 are rejected under 35 U.S.C. § 112, second paragraph, as being indefinite because they lack asserted essential steps as claimed in the process of treating neurologic damage. For the reasons set forth below, Applicant respectfully traverses the rejection.

As set forth in the Declaration of the inventor, George R. Schwartz, ¶ 7, sites of administration of each of thrombopoietin, thyroid hormone and thyrotropin are well known in the art. It is submitted that there is no requirement for a step relating to a "site of administration" unless novelty or patentability depends on such step. Such is not here the case. With respect to determination of the claimed outcome, Applicant has amended claim 1 to provide for initially determining neurological damage, and subsequently determining the effect of therapy on the neurological damage. It is submitted that such amendment overcomes the rejection under 35 U.S.C. § 112, second paragraph.

With respect to claim 19, Applicant has amended said claim to provide that the "fragment" and "variant" must have the same function, i.e., enhance platelet production. It is submitted that this overcomes the rejection on the grounds the claim is indefinite.

With respect to claim 20, Applicant submits that the use of "about" is appropriate, given that the limitation is that such amount be a therapeutically effective amount. See, e.g., *W.L. Gore & Associates, Inc. v. Garlock, Inc.*, 721 F.2d 1540, 220 USPQ 303 (Fed. Cir. 1983) (holding stretch rate defined in terms of "about" is definite where infringement could be assessed).

Authorization is given to charge payment of any additional fees required, or credit any overpayment, to Deposit Account 13-4213. A duplicate of this paper is enclosed for accounting purposes. Filed herewith is a Petition for Extension of Time to August 3, 2001, with the appropriate fee.

Attached hereto is a marked-up version of the changes made to the claims by the current amendment. The attached paper is captioned "**Version with Markings to Show Changes Made.**"

An earnest attempt has been made to respond to each and every ground of rejection advanced by the Examiner. However, should the Examiner have any queries, suggestions or comments relating to a speedy disposition of the application, the Examiner is invited to call the undersigned.

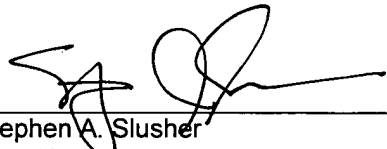
Reconsideration and allowance are respectfully requested.

Respectfully submitted,

PEACOCK, MYERS & ADAMS, P.C.

Date: August 3, 2001

By: _____


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Version with Markings to Show Changes Made

In the specification:

On page 1, line 11, delete "09/ _____" and insert in lieu thereof -09/587,552-.

This application is a continuation-in-part application of U.S. Patent Application Serial No. [09/ _____] 09/587,552 entitled "Method of Enhancement of Neurologic Recovery in Human Nervous System Damage by Use of Pharmaceutical Thrombopoietin", to George R. Schwartz, filed on June 5, 2000, which in turn is a continuation-in-part of U.S. Patent Application Serial No. 09/499,198, entitled "Method of Enhancement of Neurologic Recovery in Human Nervous System Damage by Use of Pharmaceutical Thrombopoietin," to George R. Schwartz, filed February 7, 2000, now abandoned, and which claimed the benefit of U.S. Provisional Application Serial No. 60/150,040, entitled "Method of Enhancement of Neurologic Recovery in Human Nervous System Damage by Use of Pharmaceutical Thrombopoietin", to George R. Schwartz, filed August 20, 1999. The specification of each of the foregoing is incorporated herein by reference.

On page 7, lines 9-11, delete the phrase "Ahlgren SC, Wallace H, Bishop J, Neophytou C, Raff MC: Effects of thyroid hormone on embryonic oligodendrocyte precursor cell development in vivo and in vitro. *Mol Cell Neurosci* 1997;9(5/6);420-32;".

Regulatory agent: Includes any substance which, when administered to a mammal, results in the direct or indirect alteration of cell division rates and induction of differentiation, specifically of oligodendrocyte cells. Regulatory agents include thyroid hormone, thyrotropin and the like. The effort of these regulatory agents are described generally in Rodriguez-Pena A: Oligodendrocyte development and thyroid hormone. *J. Neurobiol* 1999, Sep. 15;40(4):497-512; Ahlgren SC, Wallace H, Bishop J, Neophytou C, Raff MC: Effects of thyroid hormone on embryonic oligodendrocyte precursor cell development in vivo and in vitro. *Mol Cell Neurosci* 1997;9(5/6);420-32; Gao FB, Apperly J, Raff M: Cell-intrinsic timers and thyroid hormone regulate the probability of cell-cycle withdrawal and differentiation of oligodendrocyte precursor cells. *Dev Biol* 1998 May 1;197(1):54-66; [Ahlgren SC, Wallace H, Bishop J, Neophytou C, Raff MC: Effects of thyroid hormone on embryonic oligodendrocyte precursor cell development in vivo and in vitro. *Mol Cell Neurosci* 1997;9(5/6);420-32;] and Durand B, Raff M: A cell-

intrinsic timer that operates during oligodendrocyte development. Bioessays 2000 Jan; 22(1):64-71. The thyroid hormone, thyrotropin or the like may be isolated from a mammal, made by synthetic means, made by recombinant means, or made by any means known in the art. The regulatory agent may further be present in a formulation including one or more carriers or excipients.

On page 8, line 18, delete the word "immunogloulins" and insert in lieu thereof -immunoglobulins-.

Briefly, dosage formulations of the materials of the present invention are prepared for storage or administration by mixing the compound having the desired degree of purity with physiologically acceptable carriers, excipients and/or stabilizers. Such materials may include buffers such as phosphate, citrate, acetate and other organic acid salts; antioxidants such as ascorbic acid; low molecular weight peptides such as polyarginine, proteins such as serum albumen, gelatin or [immunogloulins] immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidinone; amino acids such as glycine, glutamic acid, aspartic acid or arginine; monosaccharides, disaccharides and other carbohydrates including cellulose or its derivatives, glucose, mannose or dextrans; chelating agents such as EDTA; sugar alcohol such as mannitol or sorbitol; counter-ions such as sodium and/or non-ionic surfactants such as Tween, Pluronics or polyethylineglycol. The TPO may be administered as the free acid or base form or as a pharmaceutically acceptable salt.

On page 12, in the table following line 10, delete the words "Table 2" and insert in lieu thereof

-Table 3-.

[Table 2] Table 3	
Days Following Birth	Administration
80	0.5 cc i.p. of a solution containing 1 µg/cc of thrombopoietin
84	0.5 cc i.p. of a solution containing 1 µg/cc of thrombopoietin
86	0.6 cc i.p. of a solution containing 1 µg/cc of thrombopoietin
88	0.5 cc i.p. of a solution containing 1 µg/cc of thrombopoietin
92	Synthroid®, 0.1 mg in 4 ounces of drinking water, ad libitum through remaining life

In the claims:

Please amend claims 1 and 19 to read as follows:

1. (First Amended) A method of treatment of neurologic damage in a mammal, comprising the steps of:

determining the extent of neurologic damage in a mammal;

administering therapeutically effective amounts of thrombopoietin to the mammal; and

monitoring the extent of neurologic damage in the mammal following the administration of thrombopoietin.

19. (First Amended) The method of claim 1 wherein said thrombopoietin is selected from the group consisting of human thrombopoietin, a fragment of human thrombopoietin enhancing production of platelets, and a variant polypeptide of human thrombopoietin enhancing production of platelets.

ATTACHMENT A

CURRICULUM VITAE

GEORGE R. SCHWARTZ, M.D.



EDUCATIONAL BACKGROUND:

- 1959-63 Hobart College, Geneva, New York, BS, Chemistry (Honors)
- 1963-67 Downstate Medical Center, State University of New York, Brooklyn, New York, Magna Cum Laude, Alpha Omega Alpha
- 1967-68 King County Hospital, Seattle, Washington; Internship (Rotating) Concomitantly Instructor, Department of Medicine, University of Washington School of Medicine
- 1968-69 Hillside Hospital, Glen Oaks, New York; Residency: Psychiatry
- 1971-72 Indiana University Medical Center, Indianapolis, Indiana; Residency: Surgery

MILITARY SERVICE:

- 1969-71 Flight Surgeon, USAF, Captain

BOARD CERTIFICATION:

- 1979 American Board of Family Practice
- 1985 American Board of Emergency Medicine
- 1995 Recertification ABEM

LICENSURE:

New Mexico ♦ New York ♦ California ♦ Pennsylvania ♦ New Jersey (inactive)

POSITIONS HELD:

- 1968-69 Admitting and Emergency Physician (half-time) Coney Island Hospital, Brooklyn, NY
- 1969-71 United States Air Force: Captain, USAF, Flight Surgeon, Chief Aerospace Medicine, Director/Base Medical Services, Kirtland Air Force Base, Albuquerque, New Mexico Included Aviation Medicine, Occupational Medicine, Lasers, Radiation. Responsible for pilot health in multi-faceted air-research base. (F-4, 104, 105, 15, B-52, helicopters, C-29)

- 1970-71 Emergency Physician Presbyterian Hospital, Albuquerque, New Mexico;
St. Joseph Hospital, Albuquerque, New Mexico
- 1972-74 Director, Emergency Services; Assistant Director, Emergency Medicine Program;
Instructor in Emergency Medicine, the Medical College of Pennsylvania, Philadelphia,
Pennsylvania (Helped to develop the first Emergency Medicine Residency Program
which included establishing all teaching rotations and liasons with other clinical
departments. Director Emergency Department Level One Trauma Center with patient
visit of 40,000 to 50,000 per year.)
- 1974-76 Director, Emergency Medicine, West Jersey Hospital (three divisions), Co-Director,
Northeastern Emergency Medical Services, Instructor in Emergency Medicine,
Medical College of Pennsylvania. Establishment of a democratic three hospital
Emergency Department Group with full billing systems and "TQM"
- 1976-77 Private Practice, writing, teaching and clinical research. Emergency Medicine,
Brotman Memorial Hospital, Culver City, CA, Santa Monica Hospital, Santa Monica,
CA
- 1978-83 Director, Division of Emergency Medicine; Associate Professor, Department of
Family, Community and Emergency Medicine, University of New Mexico School of
Medicine, Albuquerque, New Mexico (included direction of Emergency Department,
EMS Academy which trained all paramedics statewide, aero-medical support, and all
Student/Resident teaching. Established new residency program in Emergency
Medicine. Level One Trauma Center with more than 45,000 visits yearly.)
- 1983-85 Emergency Physician; hospital facilities throughout New Mexico
- 1985-1992 Los Alamos Medical Center Emergency Department, Los Alamos, NM (until 1990);
Emergency Medicine at other facilities; Medical Director, The Bridge Counseling
Centers, Los Alamos, Santa Fe, and Espanola, NM; Private Practice, Santa Fe, NM.
- 1992-1994 Emergency Physician, Cannon AFB Hospital, Clovis, NM, Mercy Hospital and
Medical Center, Bakersfield, CA, Delano Regional Medical Center, Delano, CA;
Gallup Indian Medical Center
- 1996- Emergency Physician, VISTA Staffing, Rehobeth Christian Medical Center, Gallup,
NM, Shiprock Regional Medical Center, Shiprock, NM, Gallup Indian Hospital,
Gallup, NM, Clovis Regional Medical Center, Sierra Medical Center (Truth or
Consequences, NM), Cibola Regional Medical Center (Grants, NM). Private Practice
(Santa Fe, NM).

ORGANIZATIONS(Charter or Founding Member):

Founding Member: American Trauma Society

Charter Member: American College of Emergency Physicians (President, NM Chapter, 1980-81)

Founding Member/First Secretary: American Academy of Emergency Medicine (Elected for two
terms. Elected chairman AAEM - PAC, 1999. Elected Board of Directors AAEM 1999.)

COMMUNITY ACTIVITIES:

1973-74	Chief of Medical Services, SCAA events, Pocono International Raceway
1974	Emergency Care Committee, Philadelphia County Medical Society, Chairman of Disaster Subcommittee
May 1974	Hospital Coordinator for the City of Philadelphia Disaster Exercise; Director, Planning Committee, Disaster Exercise - Philadelphia International Airport
1974-76	Director, Camden County Poison Center, Camden County, New Jersey
1975-77	HSA Review Committee
1979-82	Medical Consultant—New Mexico Poison Center
1979-81	Provost Committee on Gerontology Development at the University of New Mexico, Albuquerque, NM
1979	Albuquerque and Bernalillo County Medical Association Disaster and Emergency Care Committee
1980	President, New Mexico Chapter of American College of Emergency Physicians
1982	Toxicology Committee, American College of Emergency Physicians and Liason Designate
1987-Present	New Mexico Medical Society Medical/Legal Panel

SPECIAL TRAINING:

School of Aerospace Medicine, Brooks Air Force Base, Texas

Cardiopulmonary Resuscitation (CPR), Cardiopulmonary Cerebral Resuscitation (CPCR)
Instructor, Resuscitation Research Institute, Pittsburgh, PA

Aircraft Pilot, Radio Communications

FAA-Aviation Medicine Course. Designated Aviation Medical Examiner

PATENTS:

Use of a pharmaceutical agent in male impotence. Patent granted, U.S. Patent Office, 1977
Emergency Resuscitation Brain Cooling Device - Patent granted 1998

HONORS, AWARDS:

Medical School: Magna Cum Laude, Alpha Omega Alpha Honor Society
Gallup Award: 1973
Giraffe Award: 1990
Listed in Who's Who in the World, Who's Who in America, Who's Who in Medicine,
Who's Who in Science
Named to "2000 Most Influential Scientists of the 20th Century."

FELLOWSHIPS:

Fellow American College Emergency Physicians (FACEP) until 1996
Fellow American Academy of Emergency Medicine (FAAEM)

PUBLICATIONS:

Schwartz, GR: Systemic Use of Lidocaine, New England Journal of Medicine, 278:626, 1967.
Schwartz, GR: Loss of Physicians by Suicide, NEJM 276:1443-44, 1967.
Schwartz, GR: Dissecting Aortic Aneurysms, NEJM 286:780-81, 1972.
Schwartz, GR: Delivering Trauma Care, Med. World Press, pp. 10, Dec. 1, 1972.
Schwartz, GR: Acute Care Program at the Medical College of Pennsylvania, Hosp. Prac. 7:19, 1972.
Schwartz, GR: Medisystems, NEJM, 288:219, 1973.
Schwartz, GR: Flight Surgery, AMA News, pp. 5, Nov. 6, 1974.
Schwartz, GR: Man Power Present, Hos. Tribune, pp. 11, Jan. 1974.
Schwartz, GR: Political Problems in Emergency Medicine, Proceedings of the First Conference of Program Directors in
Emergency Medicine, ACEP, Pub. 6, 1973.
Nucleus (USAF Publication) Drugs and Drug Abuse, 9:26, p. 2, 1979; 9:27, p. 4, 1970.
Biological Clocks, 9:10, pp. 3-4, 1970.
The Common Cold, 9:30, p. 8, 1970.
Wagner, DK, Schwartz, GR: Stab wound of the Neck, Emergency Medicine, 5:216-18, May 1973.
Wagner, DK, Schwartz, GR: Prolonged Epistaxis Following Nasal Trauma, Emergency Medicine, 5:192-195, June
1973.
Wagner, DK, Schwartz, GR: Pain in the Neck after Falling, Emergency Medicine, 5:157-59, July 1973.
Wagner, DK, Schwartz, GR: Jaw Injuries, Emergency Medicine, 5:60-62, August 1973.
Wagner, DK, Schwartz, GR: Blow from an Handlebar, Emergency Medicine, 5:10, October 1973.
Wagner, DK, Schwartz, GR: Dorsiflexion Injury to the Wrist, Emergency Medicine, 5:12, December 1973.
Wagner, DK, Schwartz, GR: Penetrating Wounds of the Abdomen, Emergency Medicine, 6(1):231-33, Jan.
1974.
Wagner, DK, Schwartz, GR: Injury to the Lips and Oral Mucosa, Emergency Medicine, 6(3):278-79, March
1974.

Wagner, DK, Schwartz, GR: Injuries to the Teeth, *Emergency Medicine*. 6(4):298-99, April 1974.

Wagner, DK, Schwartz, GR: Rib Fractures, *Emergency Medicine*. 6:5, May 1974.

Wagner, DK, Schwartz, GR: Laceration of the Face, *Emergency Medicine*. 6(6):211-216, June, 1974.

Wagner, DK, Schwartz, GR: A Knife in the Back, *Emergency Medicine*, 6(8):127-28, August, 1974.

Wagner, DK, Schwartz, GR: The Abused Child, *Emergency Medicine*, 6(9):93-95, Sept. 1974.

Wagner, DK, Schwartz, GR: Trauma Triggered Labor, *Emergency Medicine*, 6(11):210-213, Nov. 1974.

Wagner, DK, Schwartz, GR: Frostbite, *Emergency Medicine*, 6(11):210-213, November, 1974.

Schwartz, GR, Nauman, L: Penetrating Trauma to the Chest, Heart and Great Vessels, *JACEP*, 2:3, May-June, 1973.

Schwartz, GR, Nauman, L: Supraventricular Tachycardias, *JACEP*, 2:4, July-August, 1973.

Schwartz, GR, Nauman, L: Status Epilepticus, *JACEP*, 2:5, September-October, 1973.

Schwartz, GR, Nauman, L: Acute CVA, *JACEP*, 2:6, November-December, 1973.

Schwartz, GR, Nauman, L: Fat Embolism, *JACEP*, 3:1, January-February, 1974.

Schwartz, GR, Nauman, L: Acute Pulmonary Edema, *JACEP*, 3:2, March-April, 1974.

Schwartz, GR: Acute Pancreatitis, *JACEP*, 3:4, July-August 1974.

Schwartz, GR: Thyrotoxic Storm, *JACEP*, 4:1, January-February 1975.

Schwartz, GR: The Emergency Department Record, *JACEP*, 3:6, November-December 1975.

Schwartz, GR: Use of the Videotape in Emergency Medicine. *Proceedings of the University Association of Emergency Medical Services* 1973. Pub: ACEP.

Schwartz, GR, et al.: Psychological and Behavioral Responses of Hospital Staff Involved in the Care of the Critically Ill, *Critical Care Medicine*, 2:48, 1974.

Schwartz, GR: Unresolved Questions about Full Time Physicians in Emergency Medicine (editorial answer), *J. Trauma*, 14(10):892-893, October 1974.

Schwartz, GR: Psychic Numbing in the Emergency Department, *Emergency Medicine Services*, 4(1):31-32, 1975.

Schwartz, GR: Review of Rape: Victims of Crisis (book review), *JACEP* 4(1):83, January-February 1975.

Schwartz, GR: Emergency Department Administration (correspondence), *JACEP* 4(1):68-69, 1975.

Schwartz, GR: Effects of Noise Levels in Emergency Departments, *NEJM* (correspondence), 1975.

Schwartz, GR: Emergency Psychiatric Care, The Management of Mental Health Crises (book review), *JACEP*, 4(5):446, September-October 1975.

Schwartz, GR: Parasympathetic and Sympathetic Responses Related to Sudden Death and Immediate Responses to Trauma, *JACEP*, October 1977.

Schwartz, GR: Use of Viscous Xylocaine in the Differential Diagnosis of Chest Pain, *JACEP*, January 1977.

Schwartz, GR: Geriatric Emergencies, *Geriatrics*, 35(6):32-33, June 1980.

Schwartz, GR: Academic Emergency Medicine: Stagnation? *Annals of Emergency Medicine*, 9(12):646, December 1980.

Schwartz, GR: Drugs and Relationship, *Aspire*, 69:3, p. 2-6, March 1984.

Schwartz, GR: Sounding an Alarm (Editorial), *Emergency Medicine News*, 16(2), February 1994.
Schwartz, GR: Sounding Another Alarm (Editorial), *Am J Emerg Med*, 12(2):254, March-April 1994.
Schwartz, GR: Pillars of Satisfaction (Letter to Editor), *Am J Emerg Med*, 12(3): May-June 1994.
Schwartz, GR: The New Healthcare Game (Letter to Editor), *NEJM* 336:1, Jan 2, 1997.

FOOD, CHEMISTRY, AND TOXICOLOGY:

Schwartz, GR: Food Poisoning: Current Concepts, *American College of Emergency Physicians Study Guide*, 1981.
Schwartz, GR: Botulism, *Uncommon Problems in Emergency Medicine*, Michael I. Greenberg, M.D., and James R. Roberts, M.D. (eds.) F.A. Davis Company, 1982, pp. 67-79.
Schwartz, GR: Botulism, *Clinical Management of Poisoning and Drug Overdose*, Haddad and Winchester (eds.) W. B. Saunders Co., 1983.
Schwartz, GR: In *Bad Taste: The MSG Syndrome*. Health Press, 1988.
Schwartz, GR: In *Bad Taste: The MSG Syndrome*. NAL/Signet, 1990.
Schwartz, GR: In *Bad Taste: The Essential Update*. Health Press, 1990.
Schwartz, GR: In *Bad Taste: The MSG Symptom Complex*. Health Press, 1999.
Schwartz, GR: Restaurant Syndromes and "Food Toxidromes," *American College of Emergency Physicians Study Course*, 1990.
Schwartz, GR: Nutrasweet and Cancer. *Western Journal of Medicine*, December 1999.

EDITORIAL:

Co-Editor and co-initiator of "Trauma Rounds," 1973-1976. (This has become a monthly feature of the medical journal, *Emergency Medicine*.)

Editor: *Geriatric Conference Series*, 1980-1982. *Geriatric Medical Journal*. Harcourt Brace Jovanovich Publishers.

EDITORIAL BOARDS:

Annals of Emergency Medicine, 1973-1981.
Resident and Staff Physician, 1978-Present.
Emergency Medical Abstracts, 1978-1985.
Medical Examination Publishing Company, 1981-1987.
Reviewer for publishing companies regarding possible new medical textbooks.
Reviewer for Health Press, 1988-Present.

BUSINESS:

Partner, Brain Resuscitation Research, LLC
Partner, Healing Research Inc.
Partner, Allied Genomics

TEXTBOOKS:

Co-author with Dan Tandberg, M.D., Emergency Medicine Continuing Education Review, Medical Examination Publishing Company, 1st ed. 1981, 2nd ed. 1985 Elsevier Publishing Company.

Schwartz, GR: Geriatric Emergencies, Brady & Co., Bowie, Maryland, 1984. (This book was co-edited with Gideon Bosker, M.D. and John Grigsby, M.D.)

Bosker G and Schwartz, GR: Geriatric Emergency Medicine. C.V. Mosby Co., 1990.

Chapters authored:

- Introduction to Geriatric Emergency Medicine
- Nutritional Emergencies
- Sepsis in the Elderly
- Family Dynamics

Schwartz, et al.: Acute Medicine and Surgery: The Essential Update, W.B. Saunders Co., 1989.

Bosker, et al.: *Emergency Medicine Therapeutics*, C.V. Mosby, 1995. Chapters authored: Food Poisoning and Anaphylaxis

Schwartz, et al: *Principles and Practice of Emergency Medicine*, 1st ed., W.B. Saunders Co., 1978, 2nd ed. W.B. Saunders Co., 1986, 3rd ed. Lea & Febiger, 1992, 4th ed. Lippincott/Williams & Wilkins, 1999.

This is a comprehensive two-volume textbook of Emergency Medicine. It has been translated into Portuguese, Japanese and other foreign rights were established. This also has been put into computer form.

Chapters authored or co-authored in First Edition:

- The Development of Emergency Medicine
- Evaluation of the Potentially Suicidal Patient
- Initial Evaluation of Acutely Injured Patients
- Trauma from Environmental Pressure Alterations
- Emergency Toxicology and General Principles of Medical Management of the Poisoned Patient
- The Work Environment

Chapters authored or co-authored in Second Edition:

- Introduction
- Abdominal Procedures
- Categorization of Emergency Care Facilities
- The Work Environment
- Evaluation of Diarrhea in the Emergency Department
- The Geriatric Patient: Overview
- Evaluation of the Potentially Suicidal Patient
- Acquired Immunodeficiency Syndrome
- Initial Evaluation of Acutely Injured Patients
- Selected Critical Pediatric Problems
- Trauma during Pregnancy
- Trauma from Environmental Pressure Alterations (Diving and Altitude Emergencies)
- Near-Drowning
- Nutritional Emergencies

Smoke Inhalation
Emergency Management of the Toxicologic Patient
Food Poisoning
Drug Abuse

Editor-in-chief, *Principles and Practice of Emergency Medicine*, 3rd Edition
This is a two volume textbook of approximately 3500 pages, Lea & Febiger, 1992

Chapters Co-authored or authored:

Introduction: Maturation of Emergency Medicine
Ethical Issues in Resuscitation
Diarrhea
Oncologic Emergencies
Pain Management in the Emergency Department
Management Issues in the Initial Treatment of Patients with Severe Trauma
Management of Head Injuries
Herpes Simplex Meningoencephalitis
Immunology in the Emergency Department: Failure of Host-Defense Mechanisms
The Sexually Assaulted Patient
Addends to Rape and Incest in Childhood and Adolescence
Pediatric Infectious Disease
Other Pediatric Emergencies
Asphyxiation, Sudden Infant Death Syndrome and Hyperpyrexia
Geriatric Emergency Medicine
Chest Pain in the Elderly
Sepsis in the Elderly
Evaluation of Falls and their Traumatic Consequences
Syncope and Mental Status Changes in the Elderly
Family Dynamics in Geriatric Crises
Diving and Altitude Emergencies
Near Drowning
Smoke Inhalation
Nutritional Emergencies
The Poisoned Patient: Overview and General Considerations
Drug Abuse
Food Poisoning
Emergency Department Management: The Work Environment
Occupational Medicine

Principles and Practice of Emergency Medicine, Fourth Edition, Williams and Wilkins, 1999. Editor in Chief and major contributor. Author or co-author of more than 50 chapters in this state-of-the-art comprehensive textbook of emergency medicine.

Pathophysiology of Dying and Reanimation
Shock
Sudden Death
Brain Death and Organ Retrieval
Ethical Issues in Resuscitation
Pain Management
Cricothyrotomy and Tracheotomy

Trans-Tracheal Ventilation
Food and Foreign Body Asphyxiation
Coin Ingestion
Imaging Techniques
Forensic Emergency Medicine
Tips for Evaluation of Trauma: Injury Patterns
Head Injuries
Spinal Injuries

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 Thoracic Trauma
 Trauma in Pregnancy
 Trauma in the Elderly
 Pediatric Trauma
 Evaluation of Chest Pain
 Acute MI and Unstable Angina
 Abdominal Pain - Evaluation
 Appendicitis
 Diverticulitis
 Viral Hepatitis
 Ano-Rectal Disorders
 Renal Failure
 Urinary Tract Infections
 The Sexually Assaulted Patient
 Patients at Higher Risk for Infection
 Fever and Sepsis
 Herpes Simplex Meningoencephalitis
 Other Infections: Osteomyelitis, Tetanus,
 Miliary TB, Coccidiomycosis,
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 Facial Pain, Trigeminal Neuralgia and Tics
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 Emergencies in Sports
 Suicidal Patient (Contribution to Sub-chapter)
 Pediatric Viral Infections
 Pediatric Dehydration, GI, Appendicitis, UTI,
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ATTACHMENT B

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Age-Dependent Penetrance of Disease in a Transgenic Mouse Model of Familial Amyotrophic Lateral Sclerosis

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The mutation gly⁸³ → ala of Cu,Zn superoxide dismutase (SOD) is found in patients with familial amyotrophic lateral sclerosis and causes motor neuron disease when expressed in transgenic mice. The progression of clinical and pathological disease was studied in a line of mice designated G1H. Clinical disease started at 91 ± 14 days of age with fine shaking of the limbs, followed by paralysis and death by 136 ± 7 days of age. Pathological changes begin by 37 days of age with vacuoles derived from swollen mitochondria accumulating in motor neurons. At the onset of clinical disease (90 days), significant death of somatic motor neurons innervating limb muscles has occurred; mice at end-stage disease (136 days) show up to 50% loss of cervical and lumbar motor neurons. However, neither thoracic nor cranial motor neurons show appreciable loss despite vacuolar changes. Autonomic motor neurons also are not affected. Mice that express wild-type human Cu,Zn SOD remain free of disease, indicating that mutations cause neuron loss by a gain-of-function. Thus, the age-dependent penetrance of motor neuron disease in this transgenic model is due to the gradual accumulation of pathological damage in select populations of cholinergic neurons.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) causes the degeneration of motor neurons in cortex, brainstem, and spinal

cord with consequent progressive paralysis and death (Mulder, 1982). Onset of disease is usually in the fourth or fifth decade of life. Initial symptoms of ALS include fasciculations, clonus, hyperreflexia, weakness in one or more limbs, or difficulty in speech and swallowing. The disease progresses rapidly with a mean survival of 3 years (95% confidence interval, 2.6–3.4 years, Emery and Holloway, 1982). Although most cases of ALS are sporadic and have an unknown etiology (Tandan and Bradley, 1985), a subset of cases have a genetic origin (Engel *et al.*, 1959). Familial cases are due to autosomal dominant inheritance of a gene that causes ALS in an age-dependent fashion (Emery and Holloway, 1982; Siddique, 1991). The constellation of clinical symptoms in familial ALS (FALS) closely parallel the sporadic disease. FALS tends to have a slightly earlier age of onset, tends to affect the legs initially, and has a more rapid course. Survival after diagnosis is only 1.5 years (95% CI, 1.1–2.0 years) for the familial disease (Emery and Holloway, 1982). The sex bias in favor of males is not seen in the genetic forms of the disease.

Approximately 10% of ALS cases are familial and of these, from 20–25% are due to mutations in the gene encoding Cu,Zn superoxide dismutase (Rosen *et al.*, 1993; Deng *et al.*, 1993). Three different forms of superoxide dismutase (SOD) are encoded in the mammalian genome (Omar *et al.*, 1992). All three enzymes contain a transition metal within their active site. This catalyzes the dismutation of superoxide (O_2^-) to hydrogen peroxide (H_2O_2).

¹ A. Y. Chiu and P. Zhai contributed equally to this work.

Cu,Zn SOD (*SOD1*) is primarily cytosolic and is expressed in every cell within the body (Crapo *et al.*, 1992). Why only motor neurons are affected by the mutations of Cu,Zn SOD described in FALS families is unknown. The mutations of Cu,Zn SOD found in affected families are primarily amino acid substitutions in structural regions of the polypeptide (Deng *et al.*, 1993; Beckman *et al.*, 1993). No deletions of the human *SOD1* gene have been described, which suggests that expression of the mutant polypeptide is required for pathogenesis.

To test the hypothesis that FALS results from a direct action of the mutant polypeptide, we and others have generated transgenic mice that express mutant forms of human Cu,Zn SOD found in affected families. Our results suggest that FALS is due to gain-of-function mutations in the enzyme. We tested the Cu,Zn SOD amino acid substitution gly⁹³ → ala in mice (Gurney *et al.*, 1994), while other groups have tested the gly⁸⁵ → arg (Ripps *et al.*, 1995) and gly³⁷ → arg mutations (Wong *et al.*, 1995). Only those mouse lines that express the mutant polypeptide at the highest levels develop motor neuron disease. In each case, the mice developed progressive paralysis of their limbs and died by 4–6 months of age. Conversely, mice that express wild-type human Cu,Zn SOD do not develop clinical motor neuron disease (Gurney *et al.*, 1994; Avraham *et al.*, 1991). Expression of wild-type human Cu,Zn SOD in mice causes mild changes at the neuromuscular junction (Avraham *et al.*, 1988, 1991) and slight vacuolar changes in the proximal axons of ventral horn motor neurons (Dal Canto and Gurney, 1995). However, it also protects mice from a range of oxidative insults including MPTP+ toxicity (Przedborski *et al.*, 1992) and ischemic damage due to stroke (Yang *et al.*, 1994).

The availability of a transgenic model for FALS now allows us to ask important questions relevant to treatment of the disease. At initial diagnosis of clinical symptoms, what is the extent of pathology in the neuraxis? If a therapy is devised that halts progression of the degenerative process, are there sufficient motor neurons remaining to allow recovery of function? To what extent does collateral sprouting compensate for the destruction of motor neurons and at what point are compensatory processes overwhelmed by the extent of damage? In patients, muscle strength and electrophysiological assessment of motor unit function have been followed over time (Dantes and McComas, 1991), yet there are no longitudinal studies of spinal cord pathology for the obvious reason that biopsy of that tissue is neither routine nor benign.

The advantage of the transgenic model is that it allows us to study the natural history of the disease from well before the onset of clinical symptoms. We compared a

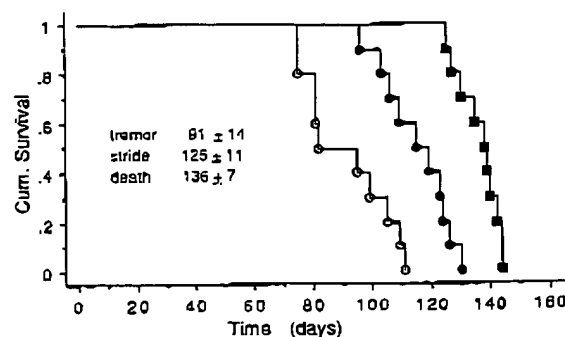


FIG. 1. Kaplan-Meier survival analysis showing the percentage of G1H mice that have not developed tremor (○), shortening of stride (□), or end-stage disease (■) at the indicated ages ($n = 10$ mice).

line of transgenic mice designated G1H that expresses mutant human Cu,Zn SOD containing a substitution of gly⁹³ → ala with nontransgenic littermates and with a second line of transgenic mice designated N29 that express comparable amounts of wild-type human Cu,Zn SOD (Gurney *et al.*, 1994). Extensive pathological damage accumulates in the spinal cord of G1H mice prior to the onset of clinical disease at 91 days of age. Despite the widespread expression of mutant human Cu,Zn SOD in the tissues of G1H mice, pathology is limited to the spinal cord, brainstem, nerve, and muscle. At end-stage disease, G1H mice are severely paralyzed and show loss of up to 50% of motor neurons in the cervical and lumbar segments of the spinal cord. Therefore, their clinical impairment is consistent with advanced motor neuron disease. Clinical disease is not seen in N29 mice expressing wild-type human Cu,Zn SOD, which indicates that mutations of *SOD1* cause motor neuron disease through a gain-of-function.

RESULTS

Clinical Disease

Clinical disease develops and progresses in a stereotyped fashion in G1H mice expressing mutant human Cu,Zn SOD. The first consistent sign of disease is a fine shaking or tremor that occurs in one or more limbs. This develops at a mean of 91 ± 14 days of age (Fig. 1). With time, the tremor becomes more pronounced and involves all of the limbs. Passive movement of the hind limbs in mice with tremor reveals increased resistance indicative of spasticity. The mice are hyperreflexive when lightly tapped on the knee or ankle. Crossed spread of both knee and ankle reflexes to the opposite limb is seen. Light

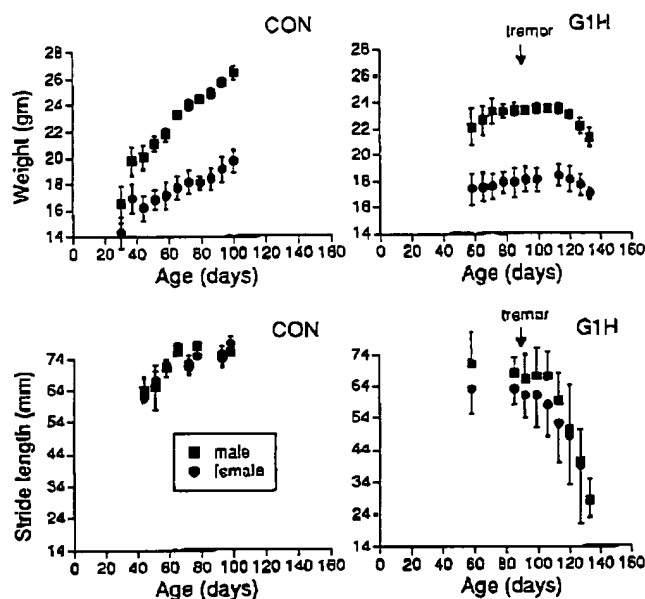


FIG. 2. Change in weight and stride length of G1H male (■) and female (●) mice with time, as compared to age-matched, nontransgenic control mice ($n = 5$ males and 5 females of each age group). The arrow indicates the mean age at onset of tremor in G1H mice (91 days).

tapping on the front of the knee also elicits repetitive, reflex movements suggestive of clonus. Roughly 2 weeks preceding the onset of tremor, G1H mice show a slowing of growth. Their weight is within the normal range for nontransgenic litter mates up to roughly 75 days of age and then plateaus thereafter (Fig. 2).

As the disease progresses, proximal muscle weakness with marked atrophy develops. Weakness and atrophy usually are more evident in the hind limbs than in the forelimbs. Mice are unable to raise their pelvis from the surface and crawl by pulling themselves with their forelimbs. As paresis becomes more pronounced, spasticity and hyperreflexia become less so, perhaps because of increasing weakness. The shaking of the limbs becomes less apparent and another, unrelated tremor of the distal joints of the toes develops which occurs in the absence of movement in the hind foot. Once paresis was evident, animals were fed with moistened food pellets placed within a petri dish on the floor of their cage. The onset of clinical weakness as assessed by shortening of stride occurred at a mean of 125 ± 11 days of age (Figs. 1 and 2). Young G1H mice had a shorter stride than age-matched N29 or nontransgenic litter mate controls, perhaps due to their smaller size (Fig. 2). Males had a slightly longer stride than females due to their larger size at all ages studied; however, the onset of clinical weakness in G1H mice did not differ between the sexes (Student's *t* test).

At end-stage disease, G1H mice are severely paralyzed and lie on their side. They generally are alert, but do not move in response to tapping on their cage or when gently prodded. When removed from their cage and placed on their side, the mice are unable to right themselves. They lose up to 10% of their body weight within the last 2 weeks of their illness (Fig. 2). Mice were euthanized when no longer able to right themselves within 30 s of being placed on their side. At end-stage disease, most mice had difficulty grooming and their fur coarsened. Roughly 40% of mice (7/18) developed infections in one or more eyes. These animals seemed unable to groom their face which may be a reflection of weakness in the forelimbs. The majority of mice (10/18) had evidence of dehydration in that their skin lost resiliency and tented or failed to flatten when pinched up and released. The mean age at which G1H mice reached end-stage disease was 136 ± 7 days of age.

Clinical disease was observed only in mice expressing mutant human Cu,Zn SOD. A cohort of four N29 mice expressing wild-type human Cu,Zn SOD at comparable levels remain free of clinical disease at 1 year of age.

Pathological Disease

We find three types of pathological changes in G1H mice. First, there are vacuolar changes in spinal motor neurons, cranial motor neurons, and motor-related areas of the brainstem. Second, cholinergic motor neurons are lost from cervical and lumbar segments of the spinal cord. This is accompanied by a reduction in the numbers of myelinated axons from the peripheral nerve. Finally, denervation and reinnervation of skeletal muscle occurs with the same time course as the loss of motor neurons from the spinal cord.

Vacuolar Changes

Vacuolation of spinal motor neurons was detectable in juvenile G1H mice as young as 37 days of age when small vacuoles were seen to accumulate in the axons of motor neurons where they traverse the descending spinal tracts and collect in the ventral roots (Fig. 3A, Table 1). Vacuolar changes invade the cell body by 45 days of age and were seen in most somatic motor neurons within the ventral horn by 69 days of age (Fig. 3B). As vacuoles filled the neuronal cell body, dendrites, and axons, they cause extensive vacuolation of the neuropil within the ventral horn.

In the spinal cord, vacuolar changes were restricted to the ventral horn; none were found in neurons within the dorsal horn or the dorsal root ganglia. Vacuolation was

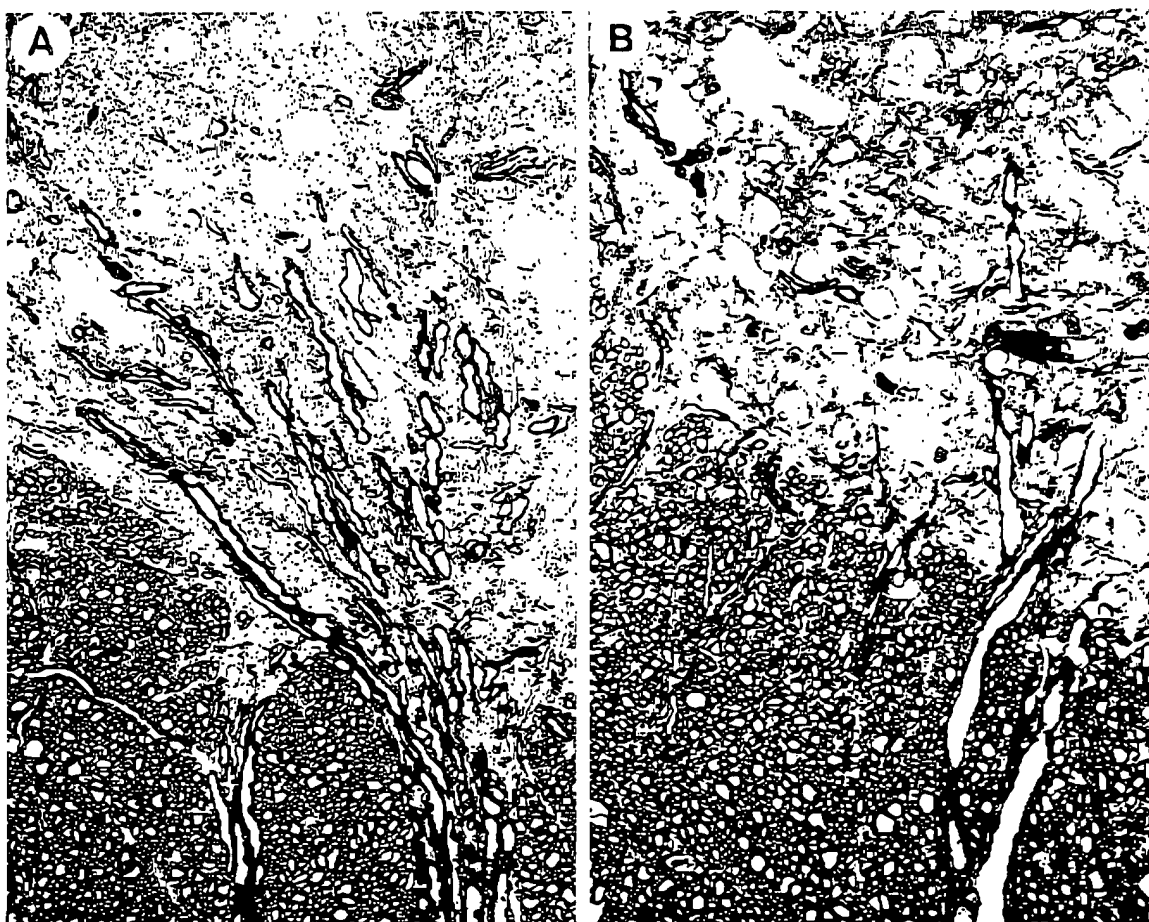


FIG. 3. (A) At 37 days of age, vacuoles begin to accumulate within the proximal axons of motor neurons in the spinal cord of G1H mice. Normal motor neurons and neuropil are seen in the upper part of the photomicrograph. However, motor axons traversing the ventral horn toward the ventral root show swelling and axoplasmic vacuolation. (B) By 78 days of age, vacuolar changes in G1H mice are present in axons, dendrites, and cell bodies of somatic motor neurons. Toluidine blue stains of 1- μ m-thick Epon embedded sections.

observed with the same timing in the cervical, thoracic, and lumbar levels of the spinal cord, although it was less pronounced in the thoracic segments (Table 1). Vacuolar changes were not observed in young, age-matched N29 mice expressing wild-type human Cu,Zn SOD. However, in older N29 mice of 247 days of age, vacuoles accumulated in the proximal axons of somatic motor neurons as seen in young adult G1H mice (Dal Canto and Gurney, 1995). These changes occurred in the absence of clinical disease.

Similar vacuolar changes in neurons and their processes also were observed in motor-related areas of the brainstem, including the hypoglossal nucleus, the dorsal nucleus of the vagus, the nucleus ambiguus, the facial nucleus, the red nucleus, and the interpeduncular nucleus. While not evident in mice examined at 37 days of

age (2/2) or at 45 days of age (2/2), these changes were fully developed by 69 days of age (2/2) (Table 2).

Cell Death

At 69 days of age, when vacuolar changes were most pronounced, counts of cholinergic motor neurons in the brainstem and spinal cord of G1H mice did not differ from those in age-matched N29 or nontransgenic mice (Figs. 4 and 5, Table 2). Loss of somatic motor neurons from the C7 and L3 segments of the spinal cord was significant by 90 days of age at the onset of clinical symptoms (Fig. 6) and worsened with age (Figs. 4 and 5). At end-stage disease, loss of motor neurons was up to 50% in these levels of the spinal cord (Fig. 5, Table 2). However, not all cholinergic motor neurons were equally af-

TABLE 1

Timing of Vacuolar Changes and Neuron Loss in Spinal Cord and Brainstem of G1H Mice Expressing Mutant Human Cu,Zn SOD

Age	35 days	45 days	69 days	102 days	End-stage 121–135 days
Vacuolation					
Spinal					
cord ^a	+/-	+	++	++	++
Brainstem ^b	-	-	-	++	++
Neuron loss					
Spinal					
cord ^c	-	-	+/-	+	++
Brainstem ^d	-	-	-	-	+/-

^a Includes cervical, thoracic, and lumbar levels.

^b Includes hypoglossal nucleus, nucleus ambiguus, and the motor component of the vagus.

^c Includes cervical and lumbar segments; neuron loss in the thoracic segment was not statistically significant.

^d Includes hypoglossal nucleus and motor component of the vagus. There was a slight, but not statistically significant, decrease in the number of hypoglossal motor neurons.

fects. Reduction in the numbers of cholinergic neurons was restricted to the ventral horn of cervical and lumbar cord. No significant reduction in somatic motor neurons innervating axial muscles occurred at thoracic (T1/T2) levels (Table 2). In the brainstem, counts of hypoglossal motor neurons showed a slight downward trend with age (Fig. 6). However, despite extensive vacuolation of these neurons, cell death did not reach statistical significance (Table 2). Autonomic motor neurons are spared in patients (Takahashi *et al.*, 1993) and that also was observed in this transgenic model. No significant reduction with age was found in two populations of preganglionic motor neurons. In the brainstem, preganglionic parasympathetic neurons of the vagus essentially were unchanged in G1H mice of 125 days of age (Fig. 5). Counts of preganglionic sympathetic neurons in the thoracic cord, identified by either ChAT or NADPH-diaphorase activity, also showed no reduction at any age studied (Fig. 6, Table 2). Thus, these results indicate that somatic motor neurons innervating the limbs were selectively vulnerable to loss, whereas other populations of cholinergic motor neurons were spared.

Loss of somatic motor neurons was restricted to G1H mice expressing mutant human SOD1; N29 mice expressing wild-type human Cu,Zn SOD had no loss of motor neurons at any age studied (Figs. 3 and 5, Table 2). There were no differences in cell counts of preganglionic neurons, hypoglossal motor neurons, or myelinated phrenic nerve axon profiles between N29 mice and nontransgenic littermate controls (Table 2).

In G1H mice, the reduction of myelinated axons in the phrenic nerve innervating the diaphragm paralleled the time course of motor neuron loss in the C7 and L3 spinal segments. The phrenic nerve innervation of the diaphragm arises from C4–C6 (Laskowski and Sanes, 1987). Counts of myelinated axons in the phrenic nerve were within the normal range at 50–69 days of age (251 ± 15 SD) in comparison to nontransgenic littermates (238 ± 10 SD) or to N29 mice (236 ± 34 SD). Loss of myelinated phrenic axons was apparent by 82–102 days of age (204 ± 39); at end-stage disease, there was only 40% of the normal complement of myelinated axons in G1H mice (95 ± 17 SD, $P < 0.0001$, Student's *t* test). Since in rats, up to 30% of the myelinated phrenic nerve axons are sensory (Langford and Schmidt, 1983), this represents a substantial loss of motor inputs to the diaphragm in G1H mice. Consistent with this observation, G1H mice became extremely sensitive to anesthesia by inhalation which may be a sign of respiratory insufficiency.

Denervation and Reinnervation

Signs of denervation and reinnervation in skeletal muscle slightly preceded the loss of motor neurons in spinal cord. At 50 days of age, no pathology was noted in the gluteus muscles of 2/2 G1H mice examined (Fig. 7). By 69 days of age, ongoing reinnervation was noted. At that age, only a small number of endplates were denervated with no inputs contacting the endplate, while up to 20% showed signs of denervation and reinnervation. Reinnervated endplates were contacted by one or more fine, unmyelinated sprouts whose origin could generally be traced to nodes of Ranvier within the intramuscular nerve. The number of endplates showing reinnervation by nodal sprouts remained a constant fraction of the total number of endplates examined throughout the course of disease (Fig. 7). As the disease progressed, the number of endplates singly innervated by myelinated axons declined. By 100 days of age, compensatory sprouting was failing to replace inputs that had been lost to disease and the number of denervated endplates increased thereafter.

Signs of denervation and reinnervation in the gluteus muscle were restricted to G1H mice. No such signs were seen in N29 mice examined between 240 and 262 days of age ($n = 4$ mice).

Somatic Tissues

Western blot analysis demonstrated expression of human Cu,Zn SOD protein in all tissues of G1H and N29 mice (Fig. 8). The order of expression of human Cu,Zn



FIG. 4. G1H mice lose ChAT-immunoreactive motor neurons in cervical levels of the spinal cord. Hemisections of C7 spinal cord, reacted to reveal ChAT-positive cells, show normal numbers of motor neurons in older nontransgenic mice (A; 160 days of age) and N29 mice expressing wild-type human Cu,Zn SOD (B; 163 days of age). Young G1H mice (C; 50 days of age) have a full complement of motor neurons prior to the onset of clinical disease, in contrast to the severe reduction seen in older G1H mice at end-stage disease (D; 123 days of age). The scale bar represents 200 μ m.

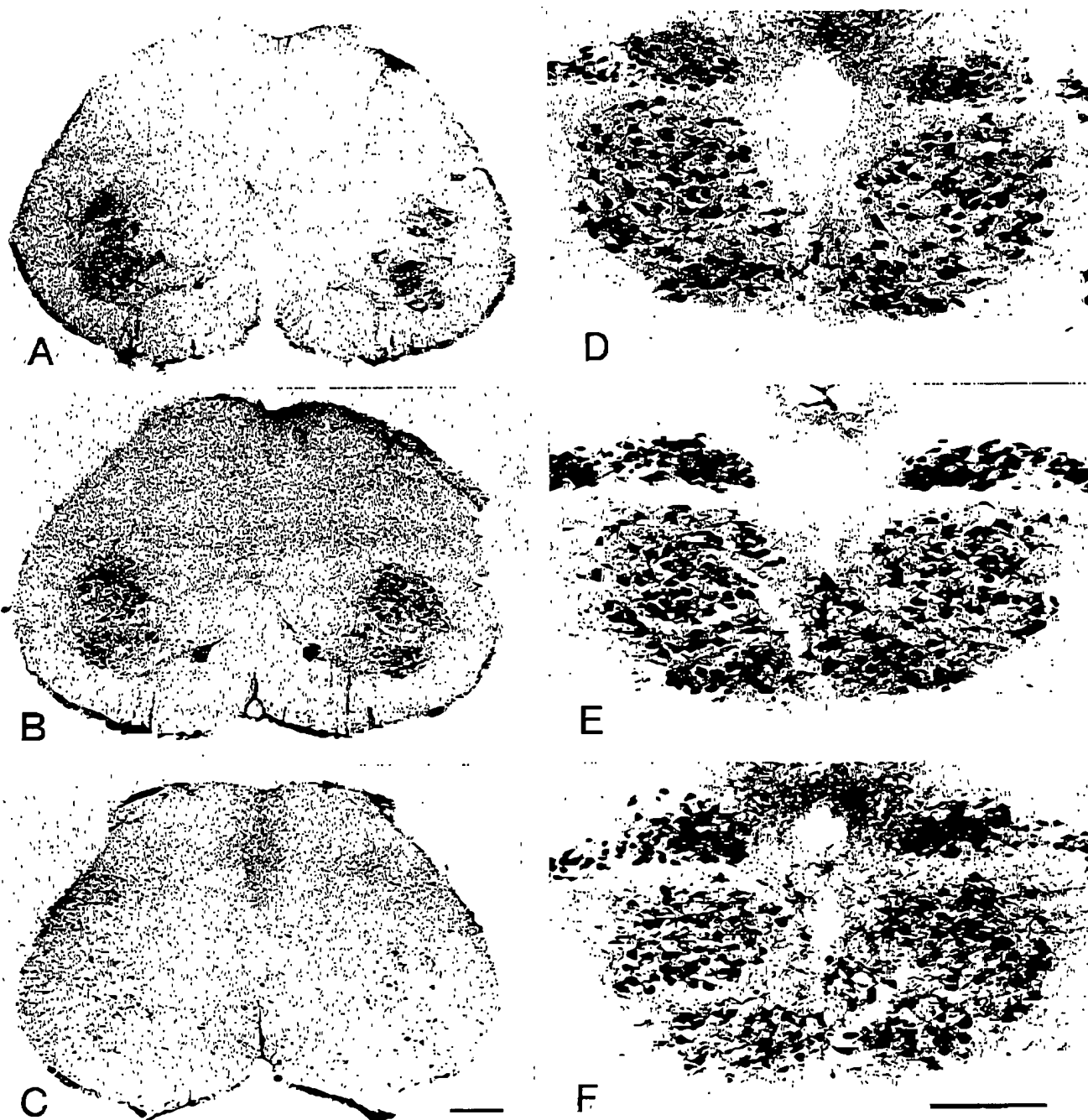


FIG. 5. Differential loss of motor neurons in G1H mice. Spinal (A–C) and cranial (D–F) motor neurons from the same animals were revealed by ChAT immunoactivity in nontransgenic mice (A and D, 160 days old), young G1H mice prior to the onset of clinical disease (B and E, 50 days old), and older G1H mice at end-stage disease (C and F, 123 days of age). Motor neurons in the lumbar (L3) levels of the spinal cord were lost as G1H mice aged. However, cholinergic neurons in the hypoglossal motor nucleus, as well as preganglionic neurons in the dorsal nucleus of the vagus, appeared relatively intact in G1H mice at end-stage disease. The scale bar represents 200 μ m.

SOD in tissues normalized to detergent extractable protein was heart, skeletal muscle > liver, kidney > brain > salivary gland, spleen, and lung.

Nontransgenic, N29 and G1H mice were euthanized for pathology at 100 days of age ($n = 2$ mice each). Both G1H animals had clinical disease at this age. The

TABLE 2

Comparison of Normalized Cell Counts in Brainstem and Spinal Cord of Transgenic Mice Expressing Mutant (G1H) or Wild-Type (N29) Human Cu,Zn SOD with Nontransgenic Mice of Similar Ages

	G1H Healthy (n = 4 mice)	G1H End-stage disease (n = 7 mice)	N29 Healthy (n = 4 mice)
Age (days)	64 ± 11	127 ± 9	168 ± 1
Spinal cord somatic motor neurons			
L3 ChAT positive	107 ± 6 (11)	55 ± 12 (11)*	100 ± 3 (13)
T1 ChAT positive	107 ± 7 (9)	92 ± 23 (8)	123 ± 12 (9)
C7 ChAT positive	92 ± 7 (10)	51 ± 17 (11)*	95 ± 3 (9)
Autonomic motor neurons			
T1 ChAT positive	105 ± 13 (7)	99 ± 39 (6)	135 ± 11 (8)
T1 diaphorase positive	98 ± 13 (10)	94 ± 6 (8)	103 ± 8 (9)
Vagal ChAT positive	98 ± 14 (7)	93 ± 22 (6)	105 ± 9 (5)
Cranial motor neurons			
XII ChAT positive	103 ± 17 (6)	89 ± 12 (6)	106 ± 13 (4)
Myelinated peripheral axons			
Phrenic nerve	104 ± 6	34 ± 3*	105 ± 4

Note. Counts were made in four "healthy" G1H mice, prior to the onset of clinical disease, and in seven G1H mice at end-stage disease. ChAT-positive cells were counted in serial sections spanning the L3, T1, and C7 spinal segments and the hypoglossal motor nucleus (XII). Depending upon the region sampled, the number of sections used for counting ranged from 4 to 13. In each population, the average number of cells per section was standardized against, and expressed as a percentage of, the average number obtained from nontransgenic, age-matched mice. Myelinated axons were counted in thin sections of the phrenic nerve. Values are mean ± SD (number of sections).

* $P < 0.005$, one-tailed Student's t test as compared to either G1H Healthy or N29 Healthy.

following tissues were examined for pathology: liver, kidney, heart, trachea, lung, salivary gland, thyroid, spleen, esophagus, stomach, duodenum, cecum, jejunum, colon, bladder, testes, epididymis, muscle, and skin. No consistent pathological findings were noted. In the kidney, infrequent vacuolar changes were noted in glomeruli. These were more marked in N29 than in G1H mice. Some mice had a mild interstitial nephritis although this was not a consistent finding between animals. Overall there was little indication of damage to either the tubular or glomerular structure. However, both N29 mice had a mild lymphocytic infiltrate. In the liver, mild biliary hyperplasia and sinusoidal dilation were noted in both G1H and N29 mice. In muscle, G1H mice had marked myodegeneration, some necrosis, variation in myofiber size, central nucleation of myofibers, mild mononuclear cell infiltrate, formation of sarcolemmal tubes, and a fatty infiltrate between myofiber bundles within fascicles. As seen in the phrenic nerve, there was marked loss of myelinated axons from intramuscular nerves. The mild pathology noted in the liver and kidney of G1H mice and the absence of consistent pathology in other tissues indicates that the disease process is restricted to brain, spinal cord, muscle, and nerve despite the widespread expression of the transgene in somatic tissues.

DISCUSSION

We generated multiple lines of transgenic mice that express, at varying levels, mutant human Cu,Zn SOD bearing the amino acid substitution gly⁹³ → ala as described previously (Gurney *et al.*, 1994). Our initial report described progressive paralytic disease developing in a single line of mice designated G1. Since that time, we have obtained two sublines of mice, both derived from the G1 line, that differ in transgene copy number. Apparently, an in-frame but out-of-register, unequal crossover event occurred within the transgene locus during meiosis and led to an increase in copy number from 18 ± 1.3 to 25 ± 1.5 (P. Zhai and M. Gurney, unpublished data). The increase in copy number occurred in an F2 male and has been stably transmitted to his progeny. To reflect the difference in copy number, the sublines were designated G1L for the original, subline with low copy number, and G1H for the newly derived subline with high copy number. Clinical disease is fully penetrant in both lines, but their survival differs. As documented in this report, G1H mice reach end-stage disease at 139 ± 12 days while G1L mice survive 170 ± 16 days (Gurney *et al.*, 1994; P. Zhai and M. Gurney, unpublished data). Clinical disease also has been seen in a second, independent line of transgenic mice designated G5. Hemizygous G5 mice remain dis-

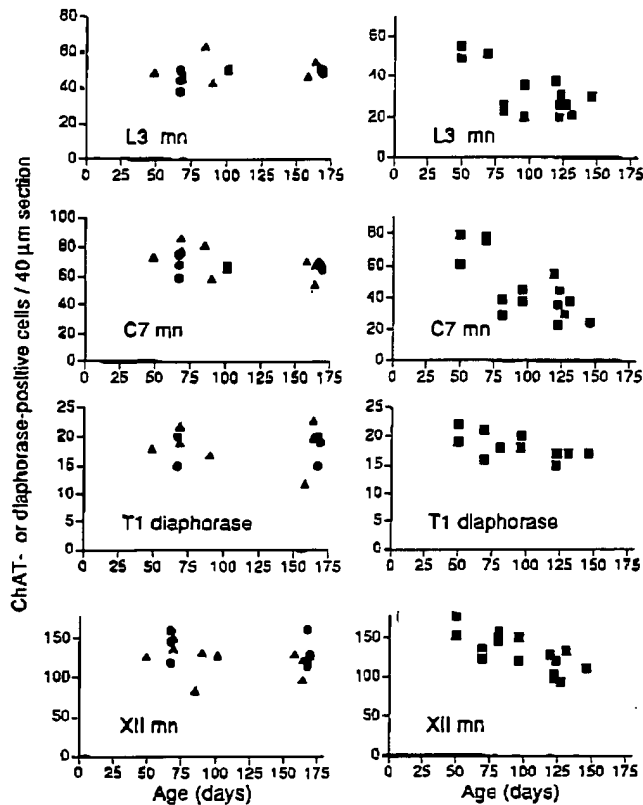


FIG. 6. Counts of ChAT- or NADPH diaphorase-positive neurons in nontransgenic (●), N29 (▲), and G1H (■) mice at the ages indicated. ChAT-immunoreactive, somatic motor neurons (mn) or diaphorase-positive preganglionic neurons were counted in serial, 40- μ m-thick sections taken through lumbar (L3), cervical (C7), and thoracic (T1) levels of the spinal cord, as well as the hypoglossal motor nucleus (XII) in the brainstem. Somatic motor neurons are reduced by up to 50% in the L3 and C7 spinal segments of G1H mice (two-tailed *t* test, $P < 0.01$), whereas the loss of hypoglossal motor neurons is gradual with increasing age (linear regression, $P < 0.01$, $r^2 = 0.587$). In contrast, there is no change in the numbers of diaphorase-positive, preganglionic neurons within thoracic cord. No differences are noted between N29 and nontransgenic animals at all ages examined.

ease-free to 600 days of age, while 92% of G5 mice bred to homozygosity (transgene copy number about 10) develop clinical disease and die by 326 ± 62 days of age (P. Zhai and M. Gurney, unpublished data). Finally, disease is weakly penetrant (about 20%) in hemizygous G20 mice which have a transgene copy number of 1.7 ± 0.6 (Dal Canto and Gurney, 1995). In all of these lines of mice, expression of mutant human Cu,Zn SOD protein is proportional to transgene copy number (Gurney *et al.*, 1994). Thus the timing and severity of clinical disease in multiple lines of mice expressing a particular mutant form of human Cu,Zn SOD correlates with transgene copy number and is independent of the site of insertion.

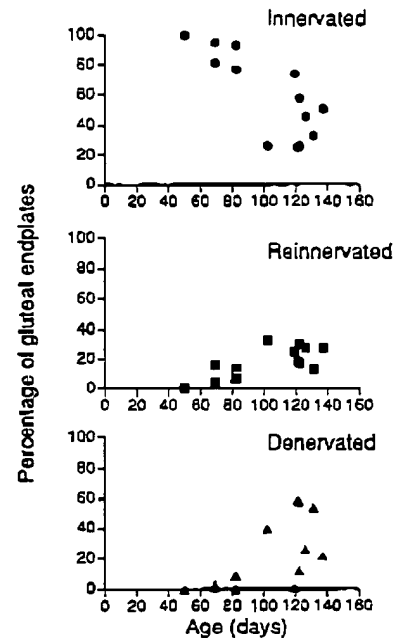


FIG. 7. The percentage of gluteal endplates that are innervated (○), being reinnervated (■), or denervated (▲) in G1H mice at different ages. Endplates scored as innervated were contacted by a single, myelinated preterminal axon. Those scored as being reinnervated were contacted by more than one unmyelinated sprout, which usually could be traced back to a node of Ranvier within the intramuscular nerve. Denervated endplates had no morphological contacts with axons.

The G1H line was chosen for in-depth study of clinical and pathological endpoints because of their short survival and the strong synchrony of disease among mice from the transgenic line. Our data suggest that the age dependence of motor neuron disease in this transgenic model is due to the gradual accumulation of pathological

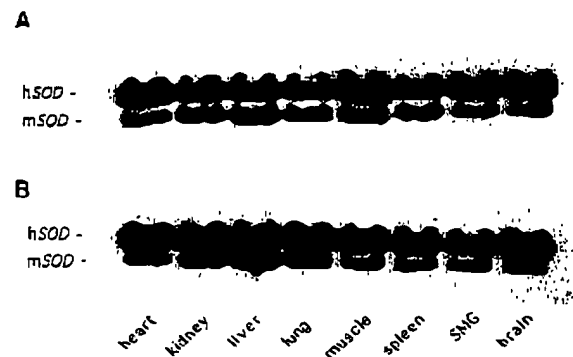


FIG. 8. Immunoblot analysis of (A) G1H and (B) N29 mouse tissues showing the pattern of human and mouse Cu,Zn SOD protein expression.

damage. The alternative model of an age-dependent event that precipitates sudden accumulation of pathological damage coincident with the onset of clinical disease is excluded by our data. Vacuolar changes occur well before the onset of clinical disease, accumulating first in the proximal axons of somatic motor neurons and extending with time into somata, dendrites, and the surrounding neuropil. They also precede the loss of motor neurons which takes place over the final 2 months of life and accompanies progressive decline in muscle strength. Motor neurons in the cervical and lumbar segments of the spinal cord are most vulnerable to loss; no significant reduction in somatic motor neurons in thoracic cord and brainstem was seen. G1H mice were compared with a second line of transgenic mice designated N29 that express wild-type human SOD1 at comparable levels. Although mild vacuolar pathology (Dal Canto and Gurney, 1994) and alterations of the neuromuscular junction (Avraham *et al.*, 1991) are seen in aged animals, the N29 line does not develop clinical disease.

The early vacuolar changes in vulnerable neurons provide an important clue to the mechanism of pathogenesis. Neuronal vacuolation is due to the swelling of endoplasmic reticulum and mitochondria (Dal Canto and Gurney, 1994). Initially, most of the changes in mitochondria are limited to the dilation of the internal cristae (Dal Canto and Gurney, 1995). These then separate and fragment as the intermembrane space becomes swollen, causing an unusual splitting of the inner and outer mitochondrial membranes with linearization of the remains of the cristae. The permeability properties of the outer mitochondrial membrane are relatively nonspecific (Benz, 1990), so it seems unlikely that the basis of the swelling is due to ionic disequilibrium. The outer mitochondrial membrane acts as a molecular sieve for hydrophilic molecules of <3000 Da, suggesting that the swelling may be due to deposition of a hydrophilic macromolecule and consequent accumulation of water. Small amounts of Cu,Zn SOD may be present within the mitochondrial intermembrane space (Weisiger and Fridovich, 1973; Liou *et al.*, 1993) and could contribute to this pathology. The vacuolar changes in endoplasmic reticulum and mitochondria might contribute to disease by impairing either energy metabolism or calcium homeostasis within vulnerable neurons.

Vacuolar changes in spinal motor neurons are detectable approximately 1 month before we can identify significant loss of neurons. While this suggests a causal relationship between the accumulation of vacuoles and cell death, vacuolation is also found in areas of the brainstem unaccompanied by significant neuronal loss. In the cervical and lumbar levels of the spinal cord, vacuoles are

found in motor neurons by 37 days of age, and significant neuronal loss occurs by 80 days of age. In contrast, we do not detect vacuolar changes in the motor-related areas of the brainstem until 69 days of age, and little neuron loss is noted in these areas at 136 days of age when the mice reach end-stage disease. Whether the differential loss of spinal and cranial motor neurons is due to a difference in vulnerability or to a difference in the timing of the disease process is not clear. Although Onuf's nucleus in the spinal cord and the oculomotor nuclei in the brainstem generally are thought to be spared in ALS, these areas develop pathology in patients whose survival is prolonged by mechanical ventilation (Hayashi and Kato, 1989). The same might be true in the transgenic model if survival was prolonged.

At end-stage disease, G1H mice showed substantial loss of cholinergic motor neurons from the cervical and lumbar segments of the spinal cord, denervation of skeletal muscle, and loss of motor inputs to the diaphragm. Our counts of cholinergic motor neurons in the C7 and L3 spinal segments allow us to estimate the rates at which these motor neurons are lost due to disease. If we assume that motor neuron loss is roughly linear with time, linear regression of the data set indicates that 0.34 ± 0.05 neurons were lost per $40\text{-}\mu\text{m}$ section per day in L3 and 0.53 ± 0.1 cells per section per day were lost in C7. The number of cholinergic motor neurons in healthy G1H mice of 50–69 days of age averaged 51.5 ± 2.8 per section in L3, and 73.4 ± 8.4 in C7. Thus, at both levels of the spinal cord, motor neurons were lost at a rate of 0.6–0.7% per day. This analysis assumes that cervical and lumbar motor neurons are all equally vulnerable to loss. However, the scatter in our data does not allow us to rule out the alternative that there may be two populations of motor neurons which are differentially vulnerable to loss. As an example of differential vulnerability, thoracic motor neurons innervating axial muscles do not show appreciable reduction in number in this transgenic model, nor did we find motor neuron loss in the nucleus of the XIIth cranial nerve. Why motor neurons innervating the limbs are particularly susceptible to loss is not clear at present.

Cell deaths during development occur primarily by apoptosis (Ellis *et al.*, 1991). The pathway of cell loss in neurodegenerative disease is not known. The hallmarks of apoptotic cell deaths are nuclear condensation and chromatin fragmentation with relative preservation of the mitochondria until late in the process of death (Kerr *et al.*, 1972; Wyllie *et al.*, 1980). We have thus far failed to find morphological evidence for nuclear condensation in the spinal cord of G1H mice, and the peculiar mitochondrial vacuolation that occurs early in disease argues

weakly against apoptosis as the pathway of motor neuron death (Dal Canto and Gurney; 1994, 1995). If apoptosis is the pathway of motor neuron death in this transgenic model, it may be difficult to detect by *in situ* labeling of DNA breaks (Gavrieli *et al.*, 1992), due to the slow loss of motor neurons over an extended period. What may be more convincing is to show that motor neurons can be rescued by coexpression of negative regulators of apoptosis such as *bcl-2* or *crmA* (Garcia *et al.*, 1992; Gagliardini *et al.*, 1994).

The loss of neurons, reduction of myelinated axons from peripheral nerves, and denervation of muscle all appear to take place with a roughly equivalent time course. We detect reinnervation in muscle about 2 weeks before significant loss of motor neurons is noted. Given that one motor neuron may innervate 20–50 gluteal muscle fibers, it should be easier to detect denervation and reinnervation than to detect a 5–10% decrease in motor neuron number. Reinnervation appears to compensate for the loss of motor neurons early in disease. The number of endplates with signs of reinnervation stays at about 20–25% throughout the course of the disease. Endplates undergoing reinnervation are detected as early as 69 days of age, while denervated endplates do not occur in appreciable numbers until after the onset of clinical symptoms at 90 days of age. Eventually, loss of motor neurons appears to overwhelm compensatory sprouting, leading to marked denervation and myodegeneration in mice at end stage disease.

The clinical findings that we identify as the first consistent signs of disease are suggestive of the involvement of upper motor centers in the transgenic model. Mice develop spasticity, clonus, hyperreflexia, and crossed spread of spinal reflexes. The fine shaking in the limbs may be a manifestation of one or more of these changes. Although we detect significant loss of motor neurons innervating the limbs at the time of onset of clinical disease, that is unlikely to account for this constellation of findings. Instead, pathological changes in motor-related areas of the brainstem (which in primates receive collateral projections from the corticospinal tract), together with degeneration of the lateral and ventral spinal columns, may account for the tremor, spasticity, clonus and hyperreflexia seen in G1H mice at the onset of clinical disease.

Despite the widespread expression of mutant human Cu,Zn SOD in somatic tissues of G1H mice, disease is restricted to brainstem, spinal cord, peripheral nerve, and muscle. No consistent pathological changes were detected in somatic tissues with the exception of mild biliary hyperplasia in liver and mild glomerular vacuolation in kidney which were noted in both G1H and N29 mice.

At end-stage disease, mice were severely paralyzed and most showed loss of weight, signs of dehydration, and signs of respiratory insufficiency. These clinical signs are all consistent with the pathology noted in the spinal cord. Motor neurons innervating the limbs were reduced by 50% and myelinated phrenic nerve axons innervating the diaphragm also showed a severe reduction in number. Clinical impairment is thus a consequence of advanced motor neuron disease.

Why is motor neuron disease age-dependent in its penetrance in this transgenic model and also in FALS families with *SOD1* mutations? In both the murine model and in affected families, mutations of Cu,Zn SOD cause clinical disease in adults. Mean age at onset for FALS is 45 years and survival after diagnosis is roughly 1.5 years (Emery and Holloway, 1982). Similarly in the line of transgenic mice studied, age at onset is in adulthood at roughly 3 months, and survival after onset of clinical symptoms also is short. The age dependence of neurological disease might be controlled in one of three ways. First, under a stochastic model, damage may accumulate with age until it reaches a threshold for disease. Second, under a senescence model, protective mechanisms may fail with age and allow the expression of disease. For example, oxidative damage to proteins and lipids accumulates slowly with age (Stadtman, 1992), while the reserve capacities of most organ systems deteriorate in a coordinate fashion. Third, a genetic program for aging may allow the penetrance of disease. Our current data favors the first hypothesis. We find that pathological damage accumulates gradually in this transgenic model of motor neuron disease, with vacuolation of axons preceding vacuolation of the cell body, and both of these changes preceding cell death (with exceptions noted above). Clearly, these will be important hypotheses for which to devise experimental tests. These transgenic mice, which express the gly⁹³ → ala mutation of human Cu,Zn SOD, and those that express the gly⁸⁵ → arg (Ripps *et al.*, 1995) and gly³⁷ → arg (Wong *et al.*, 1995) mutations, should prove invaluable in the search for an effective treatment of this devastating neurological disease.

EXPERIMENTAL METHODS

The transgene used for expression of human Cu,Zn SOD in G1H and N29 mice consisted of an 11.7-kb genomic fragment that contained the entire human *SOD1* gene (Hallewell *et al.*, 1986) together with promoter and enhancer sequences required for transcription in mice (Epstein *et al.*, 1987), as previously described (Gurney *et al.*, 1994). The G1H line has an expansion in transgene

copy number of 40% over the G1 line described in Gurney *et al.* (1994). The line has been deposited with the Induced Mutant Resource operated by The Jackson Laboratory (Bar Harbor, ME) under the strain designation B6SJL-TgN(SOD1-G93A)1Gur. Both the G1H and N29 lines were maintained as hemizygotes by mating transgenic males with B6SJL F₁ hybrid females (The Jackson Laboratory). Transgenic progeny were identified by an enzyme immunoassay (EIA) specific for human Cu,Zn SOD (Gurney *et al.*, 1994). The content of human Cu,Zn SOD in detergent soluble protein extracts of transgenic mouse brain was 3.2 ± 0.6 and 4.3 ± 1.8 ng/ μ g protein for the G1H and N29 lines, respectively. Both lines show comparable elevation in brain of human Cu,Zn SOD protein and total SOD activity. Transgenic mice were housed in microisolator cages within a barrier facility and were seronegative for common mouse pathogens. The clinical condition of mice was monitored three times per week. At end-stage disease, G1H mice are severely paralysed and are unable to right themselves when placed on their side. In the comparative studies described below, age-matched G1H, N29 and nontransgenic animals were used.

To measure stride length, mice were trained to walk up a 75 cm long, U-shaped ramp that was inclined at one end to a height of 13 cm. A bright light at the base of the ramp provided an aversive stimulus while the top of the ramp was left in subdued light. To record their tracks, hindfeet of the mice were painted with nontoxic, poster paints and their tracks were collected on paper tape lining the floor of the ramp. Stride length was measured in the central portion of the record where the animal maintained an even walking pace. Mice tended to run a few steps when released and slowed or stopped short when they reached the top of the ramp. These extreme portions of the record were discarded. Stride length was measured manually and was defined as the distance between successive right-to-right and left-to-left footprints.

For histology, mice were anesthetized by inhalation of Metofane (Pitman-Moore, Mundelein IL) and perfused transcardially with 0.13 M sodium phosphate buffer, pH 7.5 (PB), followed by 2% glutaraldehyde for thin sectioning or with cold, freshly prepared 4% paraformaldehyde in PB. For conventional morphological studies, brain, spinal cord, dorsal root ganglia, and peripheral nerves were cut first into 2-mm sections, osmicated, and embedded in Epon for further sectioning. One-micrometer-thick sections were stained with toluidine blue and examined by light microscopy; selected fields were trimmed for ultra-thin sectioning and viewed in a JEOL electron microscope. Soft tissues for pathology were

fixed in 10% buffered formaldehyde, embedded and sectioned in paraffin, and stained with hematoxylin and eosin.

For immunohistochemistry, fixed brains and spinal cords were cryoprotected in 30% sucrose in PB, embedded and frozen in OCT, and sectioned in the transverse plane on a Reichert-Jung cryostat. Forty-micrometer-thick serial sections were maintained in order in microplate wells filled with PB and stored at 4°C until used. Every fourth section was processed for ChAT immunoreactivity using monoclonal antibody 1E6 as described previously (Chiu *et al.*, 1993). NADPH-diaphorase activity was revealed in neighboring sections as described by Wetts and Vaughn (1994). Every tenth section was stained by Nissl to assess the total cell population and to confirm the segmental level of the section.

Motor neurons bearing ChAT-immunoreactivity were counted in every fourth section of cervical (C7), thoracic (T1-T2), and lumbar (L3) segments of the spinal cord, as well as spanning the hypoglossal motor nucleus and the dorsal nucleus of the vagus in the brainstem. Spinal segments were identified by the location of spinal roots and by the characteristic morphology of the spinal cord. Since the stained sections were at least 100 μ m apart, no corrections were made for split nuclei. Mice were identified by code, and cell counts were made by investigators unaware of the genotype of the animals. In both control and transgenic mice, some neurons were more darkly stained than others. We counted all cells that were above background immunoreactivity, using the level of staining in noncholinergic cells within the same section as background. By this criterion, we might have missed surviving motor neurons that have lost all immunoreactivity for ChAT. However, when the numbers of large, ventral horn cells in Nissl-stained sections were compared with the numbers of ChAT-positive neurons in adjacent sections, no discrepancies were found (data not shown). We thus feel that surviving motor neurons bear low, but detectable, levels of ChAT. In sections of T1-T2, every fourth section also was reacted for NADPH diaphorase activity and positive cells were counted. The presence of nitric oxide synthase selectively identifies the majority of the preganglionic neurons in the intermediolateral column (Wetts and Vaughn, 1994). This provided an independent assessment of preganglionic motor neurons. Usually, 9-11 sections spanning each of the spinal segments were counted. The hypoglossal and vagal nuclei in the brainstem were assessed in 4-7 sections. For each population of neurons analyzed, the average number of stained cells per section was calculated for each animal. Since we are comparing the number of cells per section,

rather than absolute cell numbers in each population, no correction factors were used.

For combined silver and acetylcholinesterase staining, gluteus muscles were harvested from mice immediately after perfusion with 4% paraformaldehyde in PS, post-fixed, and stained were postfixed and stained as described previously (Gurney *et al.*, 1992). The muscles, mounted between two coverslips for viewing, were coded and scored without knowledge of age or genotype for the following: (1) normal endplates innervated by a single, myelinated axon, (2) denervated endplates identified by acetyl cholinesterase staining that were not contacted by an axon, and (3) endplates that were in the process of reinnervation. Endplates undergoing reinnervation were contacted by either one or more unmyelinated nodal sprouts that could be traced back to the intramuscular nerve or by two or more myelinated axons. Innervation by multiple axons occurs transiently during reinnervation and then, as in development, all but one input is eliminated as the synaptic contacts become functionally mature. At least 100 endplates were scored per muscle.

Western blot analysis was performed with a rabbit antiserum raised to mouse Cu,Zn SOD (Eric Hoffman, Cephalon, Inc., West Chester PA). Samples were fractionated on 15% sodium dodecyl sulfate-polyacrylamide minigels and 5 μ g protein was analyzed in each lane. Antibody binding to the immunoblot was detected using an ECL kit (Amersham, Inc., Rockford IL) by exposure to X-ray film. Several exposures of each blot were made. Quantitative comparisons between samples were made by film densitometry.

Statistical significance was assessed with Student's *t* test. Values given in the text and tables are mean \pm standard deviation of the mean.

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